EULAR Scleroderma Trials and Research group statement and recommendations on endothelial precursor cells

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Abstract: Systemic sclerosis (SSc) is characterised by a progressive microangiopathy that contributes significantly to the morbidity of patients with SSc. Besides insufficient angiogenesis, defective vasculogenesis with altered numbers of endothelial precursor cells (EPCs) might also contribute to the vascular pathogenesis of SSc. However, different protocols for isolation, enrichment, culture and quantification of EPCs are currently used, which complicate comparison and interpretation of the results from different studies. The aim of the European League Against Rheumatism Scleroderma Trials and Research (EUSTAR) group expert panel was to provide recommendations for standardisation of future research on EPCs. Consensus statements and recommendations were developed in a face to face meeting by an expert panel of the basic science working group of EUSTAR. The findings were: cardiovascular risk factors and medications such as statins should be described in detail. A detailed description of methods considering isolation, culture, enrichment and detection of EPCs should be given. For in vitro culture of EPCs, no protocol has been shown to be superior to another, but coating with laminin and type IV collagen would resemble most closely the situation in vivo. The endothelial phenotype should be confirmed in all in vitro cultures at the end of the culture period. We recommend using CD133, vascular endothelial growth factor type 2 receptor (VEGFR2) and CD34 in combination with a viability marker for quantification of EPCs in the blood. Finally, exact standard operating procedures for fluorescence-activated cell sorting (FACS) analysis are given that should be strictly followed. In summary, the EUSTAR recommendations will help to unify EPC research and allow better comparison between the results of different studies.

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EUSTAR statement and recommendations on endothelial precursor cells

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Abstract

Objectives: Systemic sclerosis (SSc) is characterized by a progressive microangiopathy that contributes significantly to the morbidity of SSc patients. Besides insufficient angiogenesis, defective vasculogenesis with altered numbers of endothelial precursor cells (EPCs) might also contribute to the vascular pathogenesis of SSc. However, different protocols for isolation, enrichment, culture and quantification of EPCs are currently used, which complicate comparison and interpretation of the results from different studies.
Methods: The aim of the EUSTAR expert panel was therefore to provide recommendations for standardization of future research on EPCs. Consensus statements and recommendations were developed in a face-to face meeting by an expert panel of the basic science working group of EUSTAR.
Results: Cardiovascular risk factors and medications such as statins should be described in detail. A detailed description of methods considering isolation, culture, enrichment and detection of EPCs should be given. For in vitro culture of EPCs, no protocol has been shown to be superior to another, but coating with laminin and type IV collagen would resemble most closely the situation in vivo. The endothelial phenotype should be confirmed in all in vitro cultures at the end of the culture period. We recommend using CD133, VEGFR2 and CD34 in combination with a viability marker for quantification of EPCs in the blood. Finally, exact standard operating procedures for FACS analysis are given that should be strictly followed.
Conclusions: The EUSTAR recommendations will help to unify EPC research and allow better comparison between the results of different studies.

Introduction

The first histopathological hallmark of SSc is apoptosis of endothelial cells and subsequent vasculopathy. The vasculopathy in SSc patients results in a decreased capillary blood flow, which can manifest clinically as finger tip ulcers [1]. Despite the presence of several stimuli that induce the formation of new vessels such as tissue hypoxia and increased levels of vascular endothelial growth factor (VEGF), appropriate vessel formation does not occur in SSc patients [2, 3].

New vessels can be formed by angiogenesis and vasculogenesis. Angiogenesis is defined as sprouting of fully differentiated endothelial cells from preexisting vessels. In contrast, vasculogenesis describes the formation of new vessels de novo by circulating progenitor cells. Accumulating evidence suggests that vasculogenesis occurs in the adult and that endothelial precursor cells (EPCs) play an important role for the homeostasis of the vascular network. EPCs might not only be involved in the formation of new vessels in ischemic tissues, but might also contribute to the repair of preexisting vessels [4, 5]. Thus, EPCs might be interesting candidates for novel therapeutic approaches. This is indirectly supported by a recent study indicating that stem cell transplantation might improve microvascular disease in SSc [6-8]. Furthermore, EPCs could also serve as biomarkers for the individual capacity for vascular repair, new vessel formation and cardiovascular prognosis [9]. Recent studies suggest that defective vasculogenesis might also contribute to the vascular pathogenesis of SSc [1].

However, different protocols for isolation, enrichment, culture and quantification of EPCs are currently used. This complicates the interpretation of the results and might even lead to contradictory results. We summarize herein current pitfalls of EPC research and propose recommendations for standardization of future research on EPCs. These recommendations are on the level of expert opinion and were generated during a face-to face meeting of the basic science working group of EUSTAR (EULAR Scleroderma Trials And Research group). The aim of EUSTAR is to foster the awareness, understanding and research of scleroderma and its management throughout Europe [10]. This includes clinical [11] and basic science [12] research in SSc. The following recommendations were also approved by the EULAR Standing Committee for International clinical Studies Including therapeutic Trials (ESCISIT). They might not only be of interest for EPC research in SSc, but also for other rheumatic diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), in which perturbed vasculogenesis has also been demonstrated [13-19].

EPCs became important: Summary of current knowledge about EPCs in SSc

Kuwana et al. first investigated, whether vasculogenesis is impaired in patients with systemic sclerosis [20]. In this study, CD34-positive cells were enriched from peripheral-blood mononuclear cells by an immunomagnetic technique with a monoclonal antibody to CD34 coupled to magnetic beads. EPCs were defined as circulating cells positive for CD34, CD133, and the type 2 receptor for VEGF (VEGFR2) and quantified by three-colour flow cytometry. Using this definition and method of detection, the absolute numbers of EPCs were found to be lower in SSc patients than in RA patients and controls. In SSc patients, the EPC count did not correlate with the disease subset, disease duration nor the modified Rodnan Skin Score. However, the numbers of EPCs were significantly lower in SSc patients with pitting scars. Active fingertip ulcers were observed exclusively in patients with the lowest numbers of EPCs. More than 80 % of the adherent, CD133 and VEGFR2 positive cells expressed also the endothelial cell markers CD31 and the angiopoietin receptor Tie-2 and took up acetylated LDL. In contrast, the expression of markers for mature endothelial cells such as vascular-endothelium cadherin (VE-cadherin), CD146 and, von Willebrand factor was faint or lacking. In a follow-up study using the same techniques, the authors showed an increase of circulating EPCs by atorvastatin treatment in patients with SSc. However, the impaired functional capacity of EPCs in SSc patients was not improved by atorvastatin [21].

Del Papa et al. identified EPCs in whole blood by expression of CD34, VEGFR2 and CD133 with FACS [22]. Despite using the same surface markers (with, however, antibodies from a

different manufacturer), the results were different and EPCs numbers were found significantly increased. Further subgroup analysis showed a negative correlation between the number of EPCs and disease duration. Apart from disease duration, no significant correlations were observed between EPC count and clinical parameters, in particular with digital ulcers. In addition to circulating EPCs, Del Papa et al determined also the number of EPCs in the bone marrow. The numbers of cells positive for CD133 was significantly decreased in SSc. To evaluate the ability of bone marrow cells enriched for CD133 positive cells to differentiate into endothelial cells, cells were cultured in M199 medium supplemented with 10% FBS, VEGF, basic fibroblast growth factor (bFGF), and insulin-like growth factor 1 (IGF-1) on fibronectin coated flasks. In a second step, cells were purified using fluorescein isothiocyanate (FITC)-labelled agglutinin-1 and anti-FITC coated magnetic beads. After 21 days of cultures, colonies obtained from SSc were rare and smaller than colonies obtained from healthy individuals and showed signs of senescence and stress.

Allanore et al investigated EPC counts in whole blood and potential correlations with clinical parameters in patients with SSc, OA and active RA [23]. The authors measured the numbers of cells positive for CD34 and CD133 cells, but did not analyze the expression of VEGFR2. The numbers of CD34/CD133 double positive cells were significantly higher in SSc than in patients with OA, but lower than in RA patients. The numbers of CD34/CD133 double positive cells increased in parallel with the European disease activity score. In agreement with Del Papa's results, numbers of CD34/CD133 double cells tended to be higher in early stages of SSc. In another study, this group used a method to enrich immature mononuclear cells: Negative lineage (Lin-) mononuclear cells were obtained by enrichment using a human progenitor cell enrichment cocktail. After incubation with a FcR blocking reagent, these cells were then subjected to triple labelling with anti-CD133, anti-CD34 and anti-VEGFR-2. Furthermore, 7AAD was used for viability staining. EPCs, defined as Lin-/7AAD-/CD34+/CD133+/VEGFR-2+ cells, were quantified in by cell sorting/flow cytometry and by counting late outgrowth colony-forming units (CFU). SSc patients displayed higher circulating EPC counts than controls. Lower EPC counts were associated with higher Medsger's severity scores and with digital ulcers. The number of colonies correlated with levels of EPCs validating the combination of FACS surface markers [24].

Different subpopulations of EPCs

Two main populations of EPCs with different origin, function and morphological characterisation have been identified, namely a CD14-positive and a CD14-negative subset. Short term cultures contain mainly CD14-positive cells. This subset of EPCs consists of transdifferentiated monocytes, which are capable of developing into an endothelial phenotype in certain culture conditions [25]. In contrast, long-term cultures consist of CD14-negative EPCs, which form late outgrowth cultures. The CD14-negative subset of EPCs is often referred to as "angioblast-like EPCs" and possesses a high proliferation capacity [26]. Both CD14-positive and CD14-negative EPCs can form capillary like structures in vitro. Furthermore, both subsets can mediate re-endothelialization after vessel injury, improve neovascularization and can be incorporated into vessels after short term-culture under conditions promoting their differentiation into EPCs [4].

In vitro culture of EPC

Current methods of detection and quantification of EPCs by flow-cytometry rely on the surface expression of CD34, CD133 and VEGFR2. When assessed by this method, the frequency of EPCs in the blood appears considerably low (0.01% - 0.0001% of PBMCs). The low yield obtained by the method is a major limitation for functional studies. To increase the yield of putative EPCs, in vitro outgrowth techniques are widely used. However, the protocols for culture vary remarkably (Figure 1). The vast majority of studies used one of the following three culture media: Medium 199 (Gibco) has been used for the CFU assay by Hill and co-workers without supplements besides fetal bovine serum [9]. In contrast, Shintani et al. added an undefined "endothelial cell growth

supplement" [27]. Several other studies used the so-called endothelial growth medium (EGM; Clonetics) supplemented with bovine brain extract and human epidermal growth factor. Most recently, EGM-2 (Clonetics) has been introduced as an advanced endothelial culture medium system. In contrast to EGM, EGM-2 contains well defined concentrations of VEGF-A, human fibroblast growth factor-2, human epidermal growth factor, insulin-like growth factor-1, ascorbic acid, heparin and hydrocortisone.

Apart from different culture media, different extracellular matrix proteins have been used for the coating of cell-culture dishes, which might also influence the outcome. Some studies have been performed on cells expanded on culture dishes coated with collagen, whereas others used fibronectin or gelatine coated dishes.

The different culture protocols might enrich for different cell populations leading to analysis of different cells. In the first protocol summarized in Figure 1, all cell populations of the PBMC fraction are cultured together. This implies the risk of contamination with mature circulating endothelial and monocytic cells. To minimize contamination, some authors included a pre-plating step. The rationale behind this pre-plating step is that mature endothelial cells should adhere to the culture surface, whereas EPCs should remain in suspension. In this protocol, non-adherent cells are removed after 24 to 48 hours of culture and re-plated on coated culture dishes. The re-plated cells are kept in culture for various time periods before analysis.

Besides contamination, another limitation of outgrowth techniques has recently been demonstrated. George et al. showed that the frequency of EPCs quantified by this culture method does not correlate with the number of CD34/CD133/VEGFR2 positive cells determined by flow-cytometry [28].

Enrichment techniques before FACS

Immunomagnetic enrichment of cells positive for CD34 and CD133

CD34 or CD133-positive cells may be enriched from peripheral-blood mononuclear cells by immunomagnetic techniques with monoclonal antibodies to CD34 or CD133 coupled to magnetic beads. These magnetically labelled cells are then retained on a column placed in a separator, while unlabeled cells pass through. The retained cells are then eluted after removal from the magnet.

Enrichment of lineage negative cells

Lineage negative cells can be enriched from peripheral blood after the depletion of mature haematopoietic cells (positive lineage cells). For depletion, lineage positive mononuclear cells may be magnetically labelled by using a cocktail of biotin-conjugated antibodies against a panel of so-called "lineage" antigens (CD2, CD3, CD11b, CD14, CD15, CD16, CD19, CD56, CD123, and CD235a). Lineage negative cells may also be obtained by enrichment from whole blood using a human progenitor cell enrichment cocktail. This antibody cocktail crosslinks unwanted lineage positive cells to red blood cells to form immunorosettes. This increases the density of the unwanted (rosetted) cells, such that they pellet along with the free red blood cells when centrifuged over the buoyant density medium [24].

FACS markers for EPCs

Attempts to enumerate EPCs centred on the use of antibodies to VEGFR2 (KDR) in combination with CD34 to identify EPCs. Because neither is specific for EPCs alone or together, it is unclear whether the data accurately reflect the number of true EPCs in the circulation. The advent of CD133 for use in flow cytometry provided a means for detecting primitive stem cells in the circulation. Gehling et al. demonstrated that purified CD133 positive stem cells have the capacity to differentiate into endothelial cells [29]. More recent studies have demonstrated that cells positive for CD133 and VEGFR2 in the circulation have functional properties of EPCs [30, 31]. Expression of CD45, which is a marker of differentiated haematopoietic cells, on these cells has been reported

by various groups to be positive or negative [31, 32]. Dim expression of CD45 by these cells might be the cause of this confusion; hence, the use of CD45 as a gating reagent for EPCs is not supported.

The phenotyping of EPCs requires a multicolour approach. The use of antibodies to CD133 and VEGFR2 is highly recommended to increase the specificity of the analysis. CD34 should also be added to the former combination although in some instances its lack of specificity might be confusing (Table 1).

	Definition	**"early" EPCs**	**"late" EPCs**	**Endothelial cells**	**Haematopoietic stem cells**
CD45	- type I transmembrane protein - expressed on all differentiated haematopoietic cells except erythrocytes - assists in cellular activation - activates Lck (in T cells) or Syk (in B cells)	+	-	-	+
CD14	- membrane-associated glycosylphosphatidylinositol-linked protein - expressed especially macrophages - co-receptor for the detection of bacterial lipopolysaccharide - soluble form sCD14 is secreted by the liver and by monocytes	+	-	-	-
CD34	- monomeric cell surface glycoprotein - cell-cell and cell-matrix adhesion factor - found in the umbilical cord and bone marrow as haematopoietic cells and in vascular endothelium	$+_{(low)}$/-	+	+/-	+
CD133	- 5-transmembrane protein - expressed by a subset of haematopoietic stem cells - specifically localizes to cellular protrusions	-	+	-	+
VEGF R2	- 7-transmembrane domain receptor - promotes the mitogenic and angiogenic activity of VEGF	+/-	+	+	-
CD144	- also called VE-cadherin - adhesion molecule	-	+	+	-
CD146	- cell adhesion molecule of the immunoglobulin superfamily - expressed in endothelial cells - involved in intercellular junctions	-	+	+	-

Table 1: Definition of FACS-surface markers

Issues of quality controls for FACS

Various recommendations for the detection of EPCs by flow cytometry can be made. The recommendations include:

1. The rigorous cleaning of the flow cytometer to avoid contamination of the sample by debris or residual cells from a previous analysis
2. The setting and monitoring of fluorescence detectors sensitivity.
3. The mandatory collection of a large number of events (at least 500000) to identify an adequate number of this scarce population.
4. The use of a real-time viability stain such as 7-AAD or propidium iodide. Identification and exclusion of dead cells, which are a major source of non-specific staining, improve the resolution of the assay (Figure 3).
5. The use of a blocking serum to decrease non-specific binding via Fc receptors, another source of non-specific staining.
6. The establishment of a dump channel to exclude cells not of interest from analysis, which is extremely useful.

EUSTAR recommendations for studies of EPCs in SSc

Accumulating evidence suggests that vasculogenesis occurs in the adult and might play a role for vascular homeostasis. However, in SSc, several issues remain unclear and should be in the focus of further research: (i) EPCs have only been demonstrated in vascular lesions in animal models of ischemia, but not in SSc; (ii) The mechanisms by which EPCs contribute to vascular repair and neovascularisation have not fully been elucidated. It remains to be determined, whether EPCs mediate their effects in humans independently from mature endothelial cells or whether EPCs function more as bystanders of angiogenesis; (iii) Results addressing the numbers of EPCs in the blood of SSc are controversial. The initial study suggested a profound decrease of circulating EPCs, whereas subsequent studies found increased numbers of EPCs in SSc patients. These results might be explained by different disease durations in the patient populations and by different techniques for enrichment of cells before FACS analysis. Other important confounding factor might be differences in the prevalence of co-existing cardiovascular risk factors. Several medications, in particular statins, also affect the numbers of EPCs. Thus, cardiovascular risk factors and medications should be given in detail in future studies on EPCs in SSc patients. Considering the heterogeneity of the disease and the large number of confounding factors influencing the number of EPCs, studies with small number of patients are of limited help and should be avoided in future studies.

Methodical differences likely also account for different results in EPC studies. Different surface markers, enrichment techniques and operating procedures for FACS analysis, and specific culture conditions result in the characterization of different subpopulations of EPCs. While we are aware that these different subpopulations of EPCs represent different aspects of EPC biology and that there is no clear-cut “wrong” or “correct” way of identifying “true” EPCs, this expert panel believes that some basic methodical guidelines should be followed in future EPC studies.

Papers should include a detailed description of methods considering the critical points mentioned above. For in vitro culture of EPCs, no protocol has been shown to be superior to another. However, as the contents of EGM-2 are best defined, we suggest using this medium for future experiments. Systematic studies on the different extracellular matrix components for coating of the culture dishes are not available, but we feel that coating with laminin and type IV collagen would be least artificial as they resemble most closely the vascular basal membrane. For all in vitro cultures, the endothelial phenotype should be confirmed at the end of the culture period. Regarding surface markers, we recommend evaluation of CD133, VEGFR2 and CD34 in combination with a viability marker in a multiparameter flow cytometer. Finally, standard operating procedures for FACS analysis as outlined above should be strictly followed. Our key recommendations are summarized in Table 2.

Key recommendations for research on EPCs	
General issues	• Detailed described of the methods and materials used • Detailed summary of cardiovascular risk factors and medications
FACS issues	• Determination of CD133, VEGFR2 and CD34 in combination with a viability marker in a multiparameter flow cytometer • Collection of a large number of events • Blockade of Fc receptors • Experienced investigator
Culture issues	• Use of EGM-2 medium • Coating with laminin and type IV collagen • Confirmation of the phenotype at the end of the culture period

Table 2. Key recommendations for research on EPCs

These recommendations will help to unify future EPC research in the field of SSc and also might also be of interest for other rheumatic diseases with abnormal vasculogenesis such as RA and SLE.

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All other authors did not declare a specific conflict of interest

Figure legends

Figure 1: Overview about the different protocols used for in vitro expansion of angiogenic, EPC-like cells from peripheral blood mononuclear cells.

Figures 2: Figure 2 a: FACS analysis without the use of a viability marker: the first region (R1) sorts the immature mononuclear cells, the second (R2) identifies CD34 positive cells. The third dot blot gates R1 and R2, identifying the cells positive for CD34, CD133 and VEGFR2 (EPCs). **Figure 2b:** Same sample, with the use of a viability marker (7AAD): a new region is selected (R3), sorting only the 7AAD negative cells (exclusion of dead cells). The last dot blot gating R1, R2, and R3 shows a decreased EPC number compared to the previous analysis. This accounts for a non-specific staining of dead cells in the assay performed without 7AAD.

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